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TITLE: Matrix Dependent Mechanisms Involved in Tumor Promotion in Initiated Human Mammary Epithelium by Reactive Stroma

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The mammary gland relies on stromal-epithelial interactions for proper development. Tumorigenesis occurs when these signals are misinterpreted. Here we show that stromal matrix metalloproteinases (MMPs) have distinct roles in mammary gland branching morphogenesis. MMP activity is required for normal mammary gland development since mice treated with a synthetic MMP inhibitor, GM6001, have retarded ductal development. Furthermore, 3D cultures of mammary organoids also require MMP activity to branch in response to growth factors, indicating a mammary specific response. We also show that specific MMPs are required for distinct aspects of mammary gland morphogenesis. Specifically, MMP-3 is needed for branching because MMP-3 null mice have significantly fewer branches than controls. However, the ductal length is normal. In contrast, MMP-2 is required for ductal elongation, since the ducts of MMP-2 null mice are retarded in their penetration into the fat pad. However, the branching of the MMP-2 null ducts is normal. MMP-14 is a known activator of MMP-2 and we show it is highly expressed in front of terminal end buds. Mammary glands deficient in MMP-14 grown under the kidney capsule of nude mice also have defective ductal morphogenesis. Thus, these stromal enzymes are important, and have distinct roles, in patterning the mammary gland.

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Foreword

Opinions, interpretations, conclusions and recommendations are those of the author and not necessarily endorsed by the US Army.

In conducting research using animals, the investigator adhered to the "guide for the care and Use of Laboratory Animals" prepared by the Committee on Care and use of Laboratory animals of the Institue of Laboratory Resources, national Research Council (NIH publication No.86-23, Revised 1985)

X-For the protection of human subjects, the investigator adhered to policies of applicable Federal Law 45 CFR 46

Bryony Wiseman Ph.D. June 25 2002

Note: This report contains unpublished data

Table of Contents

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Introduction

The mammary gland is a dynamic organ that undergoes cycles of proliferation, differentiation and apoptosis during an individual's life cycle, as that individual goes through puberty and pregnancies. Mechanisms exist to regulate these processes and to prevent their occurrence when not required. However disruption of these mechanisms can lead to inappropriate activation of proliferation and lead to tumorigenesis. Thus we need to elucidate the mechanisms that regulate normal breast development, in order to understand what mechanisms are appropriated by breast cancers. It is becoming clear that the stromal compartment underlying the basement membrane of the epithelium contributes both to normal epithelial development and to the promotion of carcinogenesis. In addition, matrix metalloproteinases (MMPs), which are mostly expressed by stromal cells, are important in normal development and tumorigenesis. Inappropriate activation of MMP-3 and MMP-14 activity has been shown to induce mammary tumorigenesis in mice. Here we show that MMPs are also required for normal mammary development and individual MMPs have distinct roles in the proper patterning of the mammary gland.

Body

The initial goal for this research proposal was to study the promotion of mammary epithelial tumorigenesis by stromal factors, specifically MMPs. As indicated in last years report, my investigations into that avenue did not yield consistent results. To overcome this problem we set up a collaboration with Agnes Noel who initially published this system. However, she does not see a requirement for MMP-9 or MMP-2 in this system.

In last years annual report I reported that my research had changed direction and that I had found that MMPs were required for branching morphogenesis of the mammary gland during puberty. This years report will concentrate on that research and how it has been expanded.

To determine if MMPs were required for branching morphogenesis of the mammary gland we examined the effects of inhibiting MMP activity during pubertal mammary development in two ways. We analyzed branching morphogenesis inthe forth, inguinal mammary glands of female transgenic mice that overexpress the MMP inhibitor, human TIMP-1 from a β-actin promoter (β-actin-huTIMP-1 mice). We found that the mammary ducts of mice overexpressing the huTIMP-1 transgene invaded to a lesser extent than wild type controls matched for age and estrus cycle. This indicates that MMP activity is required for ductal invasion. The ducts of the β-actin-huTIMP-1 mice took two weeks longer to reach the edges of the mammary fat pad. Therefore exogenous TIMP-1 retards, but does not completely block ductal invasion. However, TIMPs have functions that are independent of their ability to inhibit MMPs,

thus to determine if the inhibition of branching morphogenesis was due to MMP inhibition, over another TIMP function, we inhibited MMP activity in a second way. As described in last year's report mice were treated with a specific pharmacological MMP inhibitor, GM6001, during their pubertal development and branching morphogenesis of their mammary glands was assessed. We found that the ducts of mice treated with inhibitor had severely diminished ductal growth compared to the age and estrus matched mice treated with the vehicle. The pubertal growth of the mammary gland is dependent on the release of estrogen. It is possible that the treatment of the mice with GM6001 had down regulated estrogen production and thereby indirectly affected mammary gland development. To address this we measured the level of estrogen in the serum of the GM6001 treated and control mice and found no differences.

The β-actin huTIMP-1 transgenic mice and the GM6001 treated mice both had systemic, whole body inhibition of MMP activity. As described in last years report, inhibition of MMP activity in vitro directly inhibits both the ability of mammary organoids to invade into collagen and their degree of branching indicating that mammary cells directly require MMP activity to induce branch formation.

We have found that over expression of TIMP-1 or inhibition of MMP activity is inhibitory for mammary ductal development. Therefore we wanted to know if the absence of TIMP-1, which should reduce MMP inhibition, would promote mammary ductal development. To do this we analyzed the morphology of mammary glands from TIMP-1 knock out mice compared to age and estrus matched controls. We found that there were no differences in the lengths of the ducts or the number of branch points. Therefore TIMP-1 is not required for ductal invasion or branching, however, it is required for the development of normal terminal end buds (TEBs), since in the TIMP-1 knock out the TEBs are almost twice the size of wild type mice.

We performed in situ hybridization to localize MMP-2, MMP-3, MMP-9 and MMP-14 mRNA within the mammary gland during puberty. We found that MMP-2 mRNA was primarily in the stroma surrounding mammary ducts, but also to a lesser degree in the epithelium. MMP-3 was exclusively stromal and located in the stroma surrounding ducts and throughout the adipose tissue. MMP-9 was expressed in both the epithelium and the stroma to similar levels throughout the gland. There were spots of concentrated MMP-9 mRNA that may come from macrophages within the gland. Most interestingly, MMP-14 was expressed primarily within and around TEBs. MMP-14 expression was mainly stromal, but was also present in the epithelium.

To determine if the expression of these MMPs during puberty correlated with a requirement for these MMPs in branching morphogenesis of the mammary gland we looked for defects in mammary gland branching morphogenesis of mice deficient for specific MMPs. As described previously, we found that in the absence of MMP-2, mammary ducts branch normally, but are retarded when they initially begin to invade the stromal fat pad at the start of puberty. We then investigated what aspect of mammary gland development MMP-2 might be regulating. We discounted a need prior to pubertal development, and a defect in the onset of puberty (or ovarian hormone release) or the response of the mammary glands to the onset of puberty by comparing the mammary glands of 20 day old of MMP-2 -/-, +/- and +/+ mice. There were no differences in ductal length or TEB formation (an indicator of response to ovarian hormone release) between genotypes.

Therefore we investigated whether MMP-2 was need for the initial invasion of the mammary ducts. There were no gross morphological differences in TEB structure, size or association with the stroma were evident at the histological level between MMP-2 null or heterozygous mammary glands. Thus we decided to investigate if there were any differences in the properties of TEBs that lack MMP-2. No differences were found in expression of basement membrane (BM) components, collagen IV and laminin-1 (potential substrates of MMP-2). No differences were found in the proliferation rate of cells within the TEBs (which are the region of cell proliferation in the pubertal mammary gland), as assessed by BrdU incorporation. However, a TUNEL assay showed that there was almost twice the number of cells undergoing apoptosis in MMP-2 null versus heterozygous TEBs. Furthermore, a fragment of caspase-3 involved in apoptosis was detected by immunohistochemistry in the same regions of apoptosis as those detected by the tunnel assay, suggesting this apoptosis was caspase-dependent. Thus MMP-2 may be driving ductal invasion by inhibiting epithelial apoptosis.

We had previously described preliminary experiments demonstrating that MMP-14 (an important activator of MMP-2) was also required for mammary gland branching morphogenesis. This work has been expanded and a difference in the development of mammary glands from 1 day old MMP-14 null and wild type littermates when grafted under the kidney capsule of immunocompromised mice has been observed. We found that each MMP-14 -/- gland had less epithlium than its contralateral wild type gland (Students paired ttest $p<0.0007$). However, there was a lot of variability between hosts. These experiments also tell us that the requirement for MMP-14 is entirely within the mammary gland since the host mice were wild type for MMP-14. We examined the mammary gland grafts by histology and found that MMP-14 null mammary glands had increased deposition of collagen, which may inhibit the invasion of ducts into the fat pad. In addition, MMP-14 -/- TEBs had fewer cells within their TEBs,

were more disorganized and had a layer of stromal material at the front of their TEBs. These phenotypes are absent in TEBs from wild type grafts as well as from MMP-2 null mammary glands suggesting that MMP-14 has a function in the mammary gland unrelated to its activation of MMP-2. We found no difference in the proliferation index between the TEBs of MMP-14 -/- and the contralateral MMP-14 +/+ grafts.

We previously reported that MMP-3 -/- mammary glands have a reduced number of branch points compared to age and estrus matched controls, both as a factor of ductal length and in total, however, in contrast to MMP-2 null glands. Furthermore, they have a reduced number of secondary and tertiary branches.In contrast to MMP-2 -/- mammary glands, MMP-3 -/- mammary ducts are no shorter than the controls.

Therefore, we have demonstrated that MMP-2 and MMP-14 are required to assist invasion of TEBs into the stromal fat pad, MMP-3 is specifically required for side branching. Thus MMP's are not only required for mammary gland branching morphogenesis,but have dedicated roles in different aspects of branching morphogenesis.

Key Research Accomplishments

- Identified a general requirement for MMPs in mammary gland branching morphogenesis
- Demonstrated a mammary cell specific requirement for MMPs in mammary gland branching morphogenesis
- Identified MMP-2 is required for initial invasion of the mammary ducts into the fat pad, probably by inhibiting apoptosis.
- Identified MMP-14 is required for the invasion of mammary ducts into the fat pad, possibly by assisting invasion through degradation of collagen and stromal matrix and by maintaining the structure of TEBs
- Identified distinct requirement for MMP-3 in primary, secondary and tertiary side branching

Conclusions

MMPs are important both in development and tumorigenesis. We have demonstrated different roles for MMPs within the morphogenesis of the mammary gland. This indicates that they have specific functions. A role for MMPs within tumorigenesis has been clearly demonstrated in the late stages of tumorigenesis as general proteinases that allow cells to break through barriers. However evidence is also building to suggest a role for MMPs in earlier stages of tumorigenesis which indicates a more defined and subtle mode of action. That we have demonstrated that MMPs have different roles to play with very different developmental consequences pushes forward this point of view. It is not yet clear what role or roles the MMPs have. MMP-2 may inhibit apoptosis by activating survival factors or making them bioavailable, for example, by cleaving IGFBP5 to release IGFs. MMP-14 may be particularly involved in breaking down ECM barriers, but may have subtler effects such as cleaving ECM molecules like laminin-5 which can then signal and induce mammary cell migration. Possibilities for the way MMP-3 may induce side branches include: affecting Wnt signaling in the mammary gland (Wnts also regulate mammary gland side branching) by releasing β-catenin through cleavage of E-cadherin, or changing Wnt associations with ECM; releasing a fragment of E-cadherin that can induce mammary epithelial cell migration; activating or making TGF-β (which also regulates mammary gland side branching) bioavailable or breaking through matrix barriers.

With regards to breast cancer, we need to understand the process by which epithelial cells invade through matrix barriers, since that is essential for the growth and spread of tumors. Understanding how this process is regulated during a controlled developmental process, will allow us to see how this process becomes defective in breast cancer.

Note: This report contains unpublished data

Reportable Outcomes

Publications

Stromal Effects on Breast Cancer and Mammary Gland Development
Bryony Wiseman & Zena Werb (2002) Science **296** 1046-49

Matrix Metalloproteinases Regulate Distinct Aspects of Mammary Gland Branching Morphogenesis.
B.S.Wiseman, M.D.Sternlicht, L.Lund, M.Sciabica, K. Holmbeck and Z.Werb
manuscript in preparation

Platform Presentations

Matrix Metalloproteinases Regulate Distinct Aspects of Mammary Gland Branching Morphogenesis.
Bryony S. Wiseman
EMBO workshop 2002: The Invasive Growth Program. Candiolo, Italy. February 21-24 2002

Matrix Metalloproteinases Regulate Distinct Aspects of Mammary Gland Branching Morphogenesis.
Bryony S. Wiseman
Mammary Gland Biology Gordon Conference, Rhode Island, June 3-8 2001

Published Abstracts

Matrix Metalloproteinases Regulate Distinct Aspects of Mammary Gland Branching Morphogenesis.
B.S.Wiseman, M.D.Sternlicht, L.Lund, M.Sciabica and Z.Werb
EMBO workshop 2002: The Invasive Growth Program. Candiolo, Italy. February 21-24 2002

Stromally Derived Matrix Metalloproteinases Foster Mammary Tumorigenesis
B.S.Wiseman, K. Cheung and Z.Werb. 40th American Society of Cell Biology Annual Meeting (#2591), San Francisco. Dec 9-13 2000.

Roles for Stromal Matrix Metalloproteinases in Mammary Tumorigenesis and Morphogenesis.
B.S.Wiseman, M.D.Sternlicht, L.Lund, M.Sciabica and Z.Werb
VIIIth International Meeting of the Metastasis Research Society, London, UK. Sept 24-27 2000.

Stromal Metalloproteinases in Morphogenesis, Remodelling and Cancer
M.D.Sternlicht, B.S. Wiseman, M.Simian, M.J.Bissell, Z.Werb. Proc. ENDO 2000 The Endocrine Society 82nd Annual Meeting, 17 (# 57) June 23, 2000.

Matrix Metalloproteinases and Tissue Inhibitors of Matrix Metalloproteinases in the Stroma Regulate Mammary Ductal Morphogenesis. J.L. Rinkenberger, M.D. Sternlicht, B.S.Wiseman, M.Sciabica, K. Holmbeck, J. Wiesen, Z. Werb. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Abstract # Z-10. June 8, 2000.

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